PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
C07K 15/04	A1	WO 92/07002
		(43) International Publication Date: 30 April 1992 (30.04.92)

(21) International Application Number: PCT/SE91/00707

(22) International Filing Date: 22 October 1991 (22.10.91)

(30) Priority data:
9003374-7 22 October 1990 (22.10.90) SE

(71) Applicant *(for all designated States except US)*: ALFA-LAVAL AGRI INTERNATIONAL AKTIEBOLAG [SE/SE]; P.O. Box 39, S-147 00 Tumba (SE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : GUSS, Bengt, Mikael [SE/SE]; Dag Hammarskjölds väg 238 B, S-756 52 Uppsala (SE). HÖÖK, Magnus [SE/US]; 129 Stevens Hill Circle, Birmingham, AL 35244 (US). JONSSON, Hans [SE/SE]; Börjegatan 58 C, S-752 29 Uppsala (SE). LINDBERG, Kjell, Martin [SE/SE]; Kornvägen 5, S-752 57 Uppsala (SE). PATTI, Joseph [US/US]; University of Alabama at Birmingham, Department of Microbiology, UAB Station - BHSB 508, Birmingham, AL 35294 (US). SIGNÄS, Lars, Christer [SE/SE]; Hamnesplanaden 2A, S-753 23 Uppsala (SE). SWITALSKI, Lech, M. [US/US]; University of Alabama at Birmingham, Department of Microbiology, UAB Station - BHSB 508, Birmingham, AL 35294 (US).

(74) Agent: INGER, Lars, Ulf, Bosson; L&U Inger Patentbyrå AB, Garvaregatan 12, S-262 63 Ängelholm (SE).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.

Published
With international search report.

(54) Title: A COLLAGEN BINDING PROTEIN AS WELL AS ITS PREPARATION

(57) Abstract

The present invention relates to a new recombinant hybrid-DNA-molecule comprising a nucleotide sequence from *S. aureus* coding for a protein, or polypeptide, having collagen binding properties.

A COLLAGEN BINDING PROTEIN AS WELL AS ITS PREPARATION.

DESCRIPTION

Technical field

The present invention relates to a collagen binding protein as well as hybrid-DNA-molecules, e.g. plasmids or phages comprising a nucleotide sequence coding for said protein. Further the invention relates to micro-organisms comprising said molecules and their use producing said protein, as well as the synthetic preparation of said protein. In particular the invention relates to a cloned gene encoding the Staphylococcus aureus collagen binding protein, or functionally active portions thereof, vectors containing the cloned gene or parts thereof, and micro-organisms transformed by those vectors as well as the cloning of the gene which specify the biosynthesis of Staphylococcus aureus collagen binding protein (CBP) (also called the collagen receptor by Switalski et al 1989) and the use of organisms transformed with the cloned gene to produce CBP or CBP like proteins. The invention also describes the use of this gene for diagnostic purposes.

The object of the present invention is to obtain a collagen binding protein.

A further object is to obtain said protein by means of a genetic engineering technique by using e.g. a plasmid comprising a nucleotide sequence coding for said protein.

A further object is to obtain a possibility of preparing said protein by chemical synthesis.

Further objects will be apparent from the following description.

Background of the invention

WO-A1-85/05553 discloses bacterial cell surface proteins having fibronectin, fibrinogen, collagen, and/or laminin binding ability. Thereby it is shown that different bacteria have an ability to bind to fibronectin, fibrinogen, collagen, and/or laminin.

TACAAAACCA AAATTACGAA TGAACAGCAA AAAGAGTTTG TTAATAATTC
ACAAGCTTGG TATCAAGAGC ATGGTAAGGA ACAAGTGAAC GGGAAATCAT
TTAATCATAC TGTGCACAAT ATTAATGCTA ATGCCGGTAT TGAAGGTACT
GTAAAAGGTG AATTAAAAGT TTTAAAACAG GATAAAGATA CCAAGGCTCC
TATAGCTAAT GTAAAATTTA AACTTTCTAA AAAAGATGGA TCAGTTGTAA
AGGACAATCA AAAAGAAATT GAGATTATAA CAGATGCAAA CGGTATTGCT
AATATTAAAG CGTTGCCTAG TGGAGACTAT ATTTTAAAAG AAATAGAGGC
GCCACGACCG TATACATTTG ATAAGGATAA AGAATATCCG TTTACTATGA
AAGATACAGA TAATCAGGGA TATTTTACGA CTATTGAAAA TGCAAAAGCG
ATAGAAAAAA CAAAAGATGT TTCTGCTCAA AAGGTTTGGG AAGGCACTCA
AAAAGTGAAA CCAACGATTT ATTTCAAGTT GTACAAACAA GATGACAATC
AAAATACAAC ACCAGTAGAC AAAGCAGAGA TTAAAAAATT AGAAGATGGA
ACGACAAAAG TGACATGGTC TAATCTTCCG GAAAATGACA AAAATGGCAA
GGCTATTAAA TATTTAGTTA AAGAAGTAAA TGCTCAAGGT GAAGATACAA
CACCAGAAGG ATATACTAAA AAAGAAAATG GTTTAGTGGT TACTAATACT
GAAAAACCAA TCGAAACAAC ATCAATTAGT GGTGAAAAAG TATGGGACGA
CAAAGACAAT CAAGATGGTA AGAGACCAGA AAAAGTCAGT GTGAATTTAT
TGGCTAACGG GGAGAAAGTA AAAACGTTAG ACGTGACATC TGAAACAAAC
TGGAAGTACG AATTTAAAGA CTTACCGAAG TATGATGAAG GAAAGAAAAT
AGAATATACA GTGACCGAAG ATCACGTAAA AGACTACACA ACAGACATCA
ACGGTACGAC AATAACGAAC AAGTATACAC CAGGAGAGAC ATCGGCAACA
GTAACAAAAA ATTGGGATGA CAATAATAAC CAAGACGGAA AACGACCAAC
TGAAATCAAA GTTGAGTTAT ATCAAGACGG AAAAGCAACA GGAAAAACGG
CAACATTAAA TGAATCTAAT AACTGGACCC ATACGTGGAC AGGATTAGAT
GAAAAAGCAA AAGGACAACA AGTAAAATAC ACAGTCGAGG AATTAACAAA
GGTCAAAGGT TATACAACAC ATGTGGATAA CAATGATATG GGTAACTTGA
TTGTGACGAA TAAATATACG CCAGAAACAA CATCAATTAG TGGTGAAAAA
GTATGGGACG ACAAAGACAA TCAAGATGGT AAGAGACCAG AAAAAGTCAG
TGTGAATTTA TTGGCTGATG GAGAGAAAGT AAAAACGTTA GACGTGACAT
CTGAAACAAA CTGGAAGTAC GAATTTAAAG ACTTACCGAA GTATGATGAA
GGAAAGAAAA TAGAATATAC AGTGACCGAA GATCACGTAA AAGACTACAC
AACAGACATC AACGGTACGA CAATAACGAA CAAGTATACA CCAGGAGAGA
CATCGGCAAC AGTAACAAAA AATTGGGATG ACAATAATAA CCAAGACGGA
AAACGACCAA CTGAAATCAA AGTTGAGTTA TATCAAGACG GAAAAGCAAC
AGGAAAAACG GCAACATTAA ATGAATCTAA TAACTGGACC CATACGTGGA
CAGGATTAGA TGAAAAAGCA AAAGGACAAC AAGTAAAATA CACAGTCGAG

TyrGlySerTyrAsnSerPheSerIleAsnTyrLysThrLysIleThrAsnGluGlnGln
LysGluPheValAsnAsnSerGlnAlaTrpTyrGlnGluHisGlyLysGluGluValAsn
GlyLysSerPheAsnHisThrValHisAsnIleAsnAlaAsnAlaGlyIleGluGlyThr
ValLysGlyGluLeuLysValLeuLysGlnAspLysAspThrLysAlaProIleAlaAsn
ValLysPheLysLeuSerLysLysAspGlySerValValLysAspAsnGlnLysGluIle
GluIleIleThrAspAlaAsnGlyIleAlaAsnIleLysAlaLeuProSerGlyAspTyr
IleLeuLysGluIleGluAlaProArgProTyrThrPheAspLysAspLysGluTyrPro
PheThrMetLysAspThrAspAsnGlnGlyTyrPheThrThrIleGluAsnAlaLysAla
IleGluLysThrLysAspValSerAlaGlnLysValTrpGluGlyThrGlnLysValLys
ProThrIleTyrPheLysLeuTyrLysGlnAspAspAsnGlnAsnThrThrProValAsp
LysAlaGluIleLysLysLeuGluAspGlyThrThrLysValThrTrpSerAsnLeuPro
GluAsnAspLysAsnGlyLysAlaIleLysTyrLeuValLysGluValAsnAlaGlnGly
GluAspThrThrProGluGlyTyrThrLysLysGluAsnGlyLeuValValThrAsnThr
GluLysProIleGluThrThrSerIleSerGlyGluLysValTrpAspAspLysAspAsn
GlnAspGlyLysArgProGluLysValSerValAsnLeuLeuAlaAsnGlyGluLysVal
LysThrLeuAspValThrSerGluThrAsnTrpLysTyrGluPheLysAspLeuProLys
TyrAspGluGlyLysLysIleGluTyrThrValThrGluAspHisValLysAspTyrThr
ThrAspIleAsnGlyThrThrIleThrAsnLysTyrThrProGlyGluThrSerAlaThr
ValThrLysAsnTrpAspAspAsnAsnAsnGlnAspGlyLysArgProThrGluIleLys
ValGluLeuTyrGlnAspGlyLysAlaThrGlyLysThrAlaThrLeuAsnGluSerAsn
AsnTrpThrHisThrTrpThrGlyLeuAspGluLysAlaLysGlyGlnGlnValLysTyr
ThrValGluGluLeuThrLysValLysGlyTyrThrThrHisValAspAsnAsnAspMet
GlyAsnLeuIleValThrAsnLysTyrThrProGluThrThrSerIleSerGlyGluLys
ValTrpAspAspLysAspAsnGlnAspGlyLysArgProGluLysValSerValAsnLeu
LeuAlaAspGlyGluLysValLysThrLeuAspValThrSerGluThrAsnTrpLysTyr
GluPheLysAspLeuProLysTyrAspGluGlyLysLysIleGluTyrThrValThrGlu
AspHisValLysAspTyrThrThrAspIleAsnGlyThrThrIleThrAsnLysTyrThr
ProGlyGluThrSerAlaThrValThrLysAsnTrpAspAspAsnAsnAsnGlnAspGly
LysArgProThrGluIleLysValGluLeuTyrGlnAspGlyLysAlaThrGlyLysThr
AlaThrLeuAsnGluSerAsnAsnTrpThrHisThrTrpThrGlyLeuAspGluLysAla
LysGlyGlnGlnValLysTyrThrValGluGluLeuThrLysValLysGlyTyrThrThr
HisValAspAsnAsnAspMetGlyAsnLeuIleValThrAsnLysTyrThrProGluThr
ThrSerIleSerGlyGluLysValTrpAspAspLysAspAsnGlnAspGlyLysArgPro
GluLysValSerValAsnLeuLeuAlaAsnGlyGluLysValLysThrLeuAspValThr
SerGluThrAsnTrpLysTyrGluPheLysAspLeuProLysTyrAspGluGlyLysLys
IleGluTyrThrValThrGluAspHisValLysAspTyrThrThrAspIleAsnGlyThr

The invention further comprises a microorganism containing at least one hybrid-DNA-molecule according to the above. The plasmid pSAC104 in an E. coli strain TG1 has been deposited at the Deutsche Sammlung von Mikroorganismen (DSM), and has thereby been allocated the deposition number DSM 6199. The present invention provides a cloned gene encoding the CBP having improved CBP-properties as compared with native CBP which is released and purified from S. aureus cells. The gene is derivied from a S. aureus strain and inserted into a cloning vector. Cells of a procaryotic organism which have been transformed with recombinant vectors are disclosed.

The invention further provides the identification of the nucleotide sequence of the gene encoding the CBP here called the cbp-gene. The deduced amino acid sequence reveals a molecule with several distinct features resembling staphylococcal cell surface proteins.

The invention also provides a procedure for production and purification of the recombinant CBP. This is done in a way so that the molecule retains its collagen binding properties, thus this recombinant CBP resemblance the native unreleased S. aureus CBP.

The invention further provides the use of the cbp-gene for diagnostic purposes. Gene probes chosen to specifically recognize the presence of the cbp gene in clinical S. aureus isolates have been used. As an example, the correlation between the presence of CBP on the surface of S. aureus strains isolated from patient with septic arthritis could be verified by the presence of the cbp-gene n all tested strains.

Appropriate carrier proteins can be coupled to the amino acid sequence as well, such as IgG binding regions of protein A.

The invention will be described in the following with reference to the examples given, however, without being restricted thereto.

S. aureus fibronectin receptor (●, ZZFR). Panel A - binding of ^{125}I-collagen to protein coated beads as a function of time. Panel B - inhibition of binding of ^{125}I-collagen by antibodies. Attachment of ^{125}I-labeled beads to cartilage as a function of time (panel C) and inhibition of attachment of ^{125}I-labeled beads to cartilage by antibodies (panel D). In this experiment 1 ug of adhesin protein was coupled to 10^8 polystyrene beads. Control beads were coated with the same molar concentration of the fibronectin receptor. Unreacted sites on the beads were saturated with bovine serum albumin. Scanning electron microscopy of beads coated with collagen adhesin (panel E) or fibronectin receptor protein (panel F) attached to cartilage.

Figure 6: Expression constructs utilized to localize the collagen binding domain within the S. aureus collagen adhesin.

Example 1:

Cloning and identification of the cbp-gene in E.coli

In order to isolate the gene encoding S. aureus CBP two commercial available (Clontech laboratories, Inc. Palo Alto, CA, USA) S. aureus strains (strain FDA 574 and FDA 485) were tested if they bound radioactivity labelled collagen. This was done according to Switalski et al 1989. Strain 574 was found to bind collagen and therefore a gene library (obtained from the same company, cat.# XL 15016) of the same strain was screened for the expression of CBP activity. Using the suppliers protocoll (in addition to this protocoll the general work involving molecular genetic appropriate protocolls found in "Current Protocolls in Molecular Biology" Vol. 1 and 2, (edited by Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, I.G. Seidman, J.A. Smith, U. Struhl, Greene, Wiley Interscience), and "Molecular Cloning". A laboratory manual, (Maniatis, T., Fritsch, E.F. and J. Sambrook (1982) Cold Spring Harbor

to detect bound primary Fab-Fragments. After incubation for 1 h at RT the filters were washed 3 X 10 min in PBS-T. The bound labelled secondary antibodies were detected by a color reaction according to the manufacturer's instructions (Bio-Rad, Instructions for preparing the BCIP/NBT color development solution for use in the immun-blot alkaline phosphatase assay kit).

By the use the above described methods several recombinant lambda phages expressing CBP-activity could be identified and isolated.

Two of these were chosen for further studies. They were called lambda coll 1 and lambda cCOLR6A respectively.

Subcloning lambda coll 1: Purified lambda coll 1 DNA was cleaved with EcoRI and the sticky ends were filled in using Klenow fragments together with the dNTP's. The blunt ended DNA-fragments originating from the S. aureus chromosome were ligated into Sma 1 cleaved pUC 18 (Pharmacia-LKB Biotechnology, Uppsala, Sweden). After transformation into freeze competent E. coli TG1 cells recombinant clones were tested for expression of the CBP. It was found that all clones expressing CBP harboured a recombinant plasmid with an insert of approx. 4 kb. One such clone called p 16 was chosen for further studies and a schematic map of the insert in this clone is shown in Fig. 1 A.

In a similar way as lambda coll 1 two other lambda clones were generated from the screening of the genomic library. Large scale cultures of pure positives were obtained and the DNA was isolated. EcoRI digestion of the clones resulted in inserts with two different sizes. Clone 1A had an insert of 3.2 kb and 3B had an insert of 4.5 kb. The larger of the two was used for further characterization. Purified insert DNA (1.5 kb) from λ GT11 clone 3B was ligated to EcoRI digested puc18 and transformed into E. coli TB-1 cell creating subclone cCOLR6A. It was also subcloned into M13mp18/JM101 for sequencing.

structure found in staphylococcal protein A (Guss et al 1984) and FnBP A (Signäs et al 1989) as well as streptococcal protein G (Guss et al 1986) and M protein (Hollingshead et al 1986). This region is thought to mediate the binding of the protein to the cell wall. The amino acid sequence nearest to the C-terminal end consists of a long stretch of hydrophobic residues followed by some charged amino acids. This region called M is similar in structure to the C-terminal end of protein A, FnBP A, Protein G and M protein.

The predicted mol.wt of the deduced CBP is approx. 133 kd (including the postulated signal sequence, S) which is very close to the mol.wt of 135 kd reported for the native released receptor (Switalski et al 1989).

In order to construct a plasmid coding for the complete cbp-gene S. aureus FDA 574 chromosomal DNA was purified and double cleaved with Hind III/Pst 1. With the guidance of Southern Transfer experiments using a 32-P labelled oligonucleotide probe (5'-ATTAAAGCGTTGCCTAGTGG-3') it was known that cleavage with these enzymes should generate an approx. 3,2 kb fragment corresponding to the 3'end of the cbp-gene. After cleavage with these enzymes the chromosomal DNA was electophoretically separated in an agarose gel. A gel slice ruffly corresponding to right size was cut out and the DNA fragments eluted and purified. The purified fragments were ligated into pUC 18 previously double cleaved with Hind III/Pst 1. After ligation followed transformation into E. coli TG1 and the resulting recombinant clones were screened for obtaining the right fragment using colony hybridization with the same probe. One positive clone hybridizing with the radioactive probe was chosen for further studies. This clone called E. coli pSAC 100 was cleaved with Hind III and a purified approx. 1,8 kb Hind III fragment from p 16 (encoding the 5'end of the cbp-gene, Fig. 1 A) was ligated into pSAC 100. After transformation

activity. This result is in contrast with the findings reported by Switalski et al 1989 which found that purified or partly purified native collagen receptor could not inhibit the binding of collagen to S. aureus Cowan 1 cells. The conclusion of this is that recombinant CBP expressed has retained more of its original features than the released protein from the staphylococci.

Although it was possible to detect CBP activity in the recombinant E. coli lysate it was not possible to affinity purify the CBP using immobilized collagen or gelatine. Although in "Western transfer" experiments with lysates from the above mentioned recombinant clones, using the Fab-fragments described in Example 1, was it possible to detect bands corresponding to high mol.wt. fragments. These were in the same size as expected from calculations using the deduced amino acid sequence.

Example 4:

Expression and of a CBP fusionprotein which retains the collagen binding properties after purification

Been unsuccessful to affinity purify the recombinant produced CBP, using immobilized collagen, another approach was used. This approach was to fuse the cbp-gene or parts of the gene to another gene encoding a so called affinity tail (Methods in enzymology, Part 185). The affinity tail to be tested was the part from the protein A gene encoding the IgG-binding domains (Uhle'n et al 1984). Therefore a vector encoding the above mentioned domains from protein A was used. This vector called pNSEQ1, which was a gift from Dr. M. Uhle'n contains in addition to the IgG-binding domains (E, D, A, B and C) two multi cloning sites (MCS) which flank the IgG-binding domains. This makes it possible to chose a restriction enzyme that has a recognizion site in both the MCS which upon cleavage results in a release of (provided the restriction site is not present in the IgG-binding domains) a DNA fragment encoding the

Example 5:

The use of the CBP-gene as a diagnostic tool

Two oligonucleotides (JP-1,5'-AGT-GGT-TAC-TAA-TAC-TG-3' and JP-2,5'-CAG-GAT-AGA-TIG-GTT-TA-3') complementary to regions of the CBP that flanked the repeats B1, B2, and B3 were constructed (Oligo's Etc.). Genomic DNA from 6 different Staphyloccus aureus strains that were known to bind ^{125}I-collagen (Table 1) were isolated as previously described by Lindberg. Polymerase chain reaction (PCR) was performed with a Cetus/Perkin-Elmer DNA Thermocyler. Reaction mixtures (100 µl) contained 1mM of each primer, 200 mM of each dNTP, 1 mM Tris-HCl (pH 8.3), 5 mM KCl, 15 mM $MgCl_2$, 0.001% gelatin, 3 µg template DNA, and 2.5 U AmpliTaq DNA polymerase. The reaction mixtures were overlayed with 100 µl of mineral oil and amplified for 30 cycles consisting of a 2 minute denaturation at 94°C, a 2 minute annealing period at 55°C, and a 3 minute extension period at 72°C. After amplification, 15 µl of the PCR products were analyzed on a 1% agarose gel (SeaKem GTG, FMC Inc., Rockland, Maine).

PCR analysis of the genomic DNA from the different S. aureus isolates revealed two distinctly different sized products. FDA 574, Cowan, and #13 all had gene products of 1677 bp, whereas Phillips, #7, and #14391 had gene products of 1118 bp. S. aureus Newman, a known non-collagen binder had no detectable PCR product. There is a direct correlation between the repeat size and the estimated molecular weight of the purified native collagen receptor from the different S. aureus strains tested. Upon further sequence analysis, it appears that a PCR product of 1677 bp corresponds to 3 repeat units, each 560 bp long. A PCR product of 1118 bp therefore corresponds to 2 repeats, each 560 bp long. These data correlate highly with the estimated molecular weight of purified native collagen receptors of 135 kd and 115 kd respectively.

lysostaphin lysates prepared from different S. aureus isolates (Figure 3). Lysostaphin digestion releases from the cell surface of S. aureus a number of proteins, around 30 bands can be visualized in the lysates by Coomassie Brilliant Blue staining of the gel (Switalski et al., 1989). The anti-adhesin antibodies recognized a component of M_r 135 kd in the lysate of strain Cowan (Figure 3, lane a), which is in agreement with our previous observations (Switalski et al., 1989). The major immunoreactive protein detected in the lysates of the other collagen adhesin positive strains (CA+) varied in molecular weight and was present as either 110 kd or 135 kd (Figure 3, lanes b through h). No correlation was observed between the apparent size of the immunoreactive protein and the collagen binding capacity of a strain or its origin (bone, synovial fluid). None of the nine non-binding collagen S. aureus strains tested expressed an immunoreactive protein (Figure 3, lane i).

Collagen adhesin mediated attachment of staphylococci to collagenous substrata.

The relationship between the ability to express a collagen adhesin and the observed localization of an infection within collagen rich tissues prompted us to analyze the role of the cell surface adhesin in bacterial attachment to collagen containing substrates. We initially studied attachment of bacteria to surfaces coated with type II collagen. Results indicated that a collagen coated surface was an excellent attachment substrate for strains which express a surface localized collagen adhesin. The attachment is time dependent and saturable reaching an equilibrium after 3 hours of incubation (Figure 4A). The number of attaching bacteria is not influenced by the size of the adhesin since strains #14 and Phillips, which either express a 135 kd or 110 kd adhesin respectively, attached in equal numbers to the collagen coated substrate. When bacteria were preincubated with anti-adhesin antibodies, against the collagen

Creation of artificial bacteria

"Artificial bacteria" were prepared by covalently coating polystyrene beads (1.2 μm vs. staphylococci 0.8 - 1.0 μm in diameter) with the collagen adhesin protein. These beads were then tested in a series of experiments analogous to those performed with intact bacteria. The collagen adhesin (CA) coated beads, but not beads coated with a recombinant form of another staphylococcal cell surface component, the fibronectin receptor (Flock et al., 1987), bound ^{125}I-collagen (Figure 5A) in a manner similar to that of CA+ strains of S. aureus (Speziale et al., 1986). This binding was abolished by anti-CA antibodies, whereas preimmune antibodies did not effectively inhibit binding (Figure 5B). When "artificial bacteria" were assayed for the ability to attach to collagen (data not shown) or cartilage, we found that CA beads adhered to the substrate in a time dependent manner, identical to that of CA+ strains of S. aureus, while beads coated with the fibronectin receptor did not adhere at significant levels (Figure 5C). The anti-CA antibody inhibited the adhesion of CA beads to cartilage in a dose dependent fashion, whereas a preimmune antibodies had no effect (Figure 5D). Once again the quantitative binding data was corroborated by electron microscopy observations. CA coated beads attached in large numbers to cartilage tissue, in particular to collagen fibers (Figure 5E), while beads coated with the fibronectin receptor did not (Figure 5F).

Localization of the collagen binding domain within the collagen adhesin.

Various expression constructs have been created in E. coli in effort to specifically localize the collagen binding domain. Two different types of expression vectors have been utilized in these experiments, pKK223-3 and pGEX-2T, the second of which results in the collagen adhesin fused to glutathione-S-transferase. To date the smallest

including man, the protein, or polypeptide is dispersed in sterile, isotonic saline solution, optionally while adding a pharmaceutically acceptable dispersing agent. Different types of adjuvants can further be used in order to sustain the release in the tissue, and thus expose the protein or the peptide for a longer time to the immundefense system of a body.

A suitable dosage to obtain immunization is 0,5 to 5 µg of CBP, or polypeptide, per kg bodyweight and injection of immunization. In order to obtain a durable immunization, vaccination should be carried out at more than one consecutive occasion with an interval of 1 to 3 weeks, preferably at three occasions.

When using the present CBP, or polypeptide, for topical, local administration the protein is dispersed in an isotonic saline solution to a concentration of 25 to 250 µg per ml. The wounds are then treated with such an amount only to obtain a complete wetting of the wound surface. For an average wound thus only a couple of millilitres of solution are used in this way. After treatment using the protein solution the wounds are suitably washed with isotonic saline or another suitable wound treatment solution.

Further the collagen binding protein as well as the minimal collagen binding site polypeptide, of the present invention can be used to diagnose bacterial infections caused by Staphylococci strains, whereby a collagen binding protein of the present invention is immobilized on a solid carrier, such as small latex or Sepharose® beads, whereupon sera containing antibodies are allowed to pass and react with the CBP thus immobilized. The agglutination is then measured by known methods.

Further, the CBP, or the polypeptide can be used in an ELISA test (Enzyme Linked Immuno Sorbent Assay; E Engvall, Med. Biol. 55, 193, (1977). Hereby wells in a polystyrene microtitre plate are coated with the CBP, and incubated over night at 4°C. The plates are then thorough-

References:

Carret, G., H. Emonard, G. Fardel, M. Druguet, D. Herbage, and J. P. Flandrois. 1985. Ann. Inst Pasteur (Paris) 136A:241-245.

Guss, B., M. Uhle'n, B. Nilsson, M. Lindberg, J. Sjöquist and J. Sjödahl. 1981. J. Biochem., 138, 413-420.

Guss, B., M. Eliasson, A. Olsson, M. Uhle'n, A.-K. Frej, H. Jörnvall, J.-I. Flock and M. Lindberg. 1986. EMBO J., 5, 1567-1575.

Holderbaum, D., R.A. Spech and L. A. Ehrhart. 1985. Collagen Relat. Res. 5:261-271.

Holderbaum, D., G. S. Hall and L. A. Ehrhart. 1986. Infect. Immun. 54:359-364.

Hollingshead, S. K., V. A. Fischetti and J. R. Scott. 1986. J. Biol. Chem. 261:1677-1686.

Signäs, S., G. Raucci, K. Jönsson, P.-E. Lindgren, G. M. Anantharamaiah, M. Höök and M. Lindberg. 1989. Proc. Nutl. Acad. Sci. USA. 86:699-703.

Speziale, P. G. Raucci, L. Visal, L. M. Switalski, R. Timpl and M. Höök. 1986. J. Bact. 167:77-81.

Switalski, L. M., P. Speziale and M. Höök. 1989. J. Biol. Chem. 264:21080-21086.

Uhle'n, M., B. Guss, B. Nilsson, S. Gatenbeck, L. Philipsson and M. Lindberg. 1984. J. Biol. Chem. 259:1695-1702.

Vercellotti, G. M., J. B. McCarthy, P. Lindholm, P. K. Peterson, H.S. Jacob and L. T. Furcht. 1985. Am. J. Pathol. 120:13-21.

TACAAAACCA AAATTACGAA TGAACAGCAA AAAGAGTTTG TTAATAATTC
ACAAGCTTGG TATCAAGAGC ATGGTAAGGA AGAAGTGAAC GGGAAATCAT
TTAATCATAC TGTGCACAAT ATTAATGCTA ATGCCGGTAT TGAAGGTACT
GTAAAAGGTG AATTAAAAGT TTTAAAACAG GATAAAGATA CCAAGGCTCC
TATAGCTAAT GTAAAATTTA AACTTTCTAA AAAAGATGGA TCAGTTGTAA
AGGACAATCA AAAAGAAATT GAGATTATAA CAGATGCAAA CGGTATTGCT
AATATTAAAG CGTTGCCTAG TGGAGACTAT ATTTTAAAAG AAATAGAGGC
GCCACGACCG TATACATTTG ATAAGGATAA AGAATATCCG TTTACTATGA
AAGATACAGA TAATCAGGGA TATTTTACGA CTATTGAAAA TGCAAAAGCG
ATAGAAAAAA CAAAAGATGT TTCTGCTCAA AAGGTTTGGG AAGGCACTCA
AAAAGTGAAA CCAACGATTT ATTTCAAGTT GTACAAACAA GATGACAATC
AAAATACAAC ACCAGTAGAC AAAGCAGAGA TTAAAAAATT AGAAGATGGA
ACGACAAAAG TGACATGGTC TAATCTTCCG GAAAATGACA AAAATGGCAA
GGCTATTAAA TATTTAGTTA AAGAAGTAAA TGCTCAAGGT GAAGATACAA
CACCAGAAGG ATATACTAAA AAAGAAAATG GTTTAGTGGT TACTAATACT
GAAAAACCAA TCGAAACAAC ATCAATTAGT GGTGAAAAAG TATGGGACGA
CAAAGACAAT CAAGATGGTA AGAGACCAGA AAAAGTCAGT GTGAATTTAT
TGGCTAACGG GGAGAAAGTA AAAACGTTAG ACGTGACATC TGAAACAAAC
TGGAAGTACG AATTTAAAGA CTTACCGAAG TATGATGAAG GAAAGAAAAT
AGAATATACA GTGACCGAAG ATCACGTAAA AGACTACACA ACAGACATCA
ACGGTACGAC AATAACGAAC AAGTATACAC CAGGAGAGAC ATCGGCAACA
GTAACAAAAA ATTGGGATGA CAATAATAAC CAAGACGGAA AACGACCAAC
TGAAATCAAA GTTGAGTTAT ATCAAGACGG AAAAGCAACA GGAAAAACGG
CAACATTAAA TGAATCTAAT AACTGGACCC ATACGTGGAC AGGATTAGAT
GAAAAAGCAA AGGACAACA AGTAAATATAC ACAGTCGAGG AATTAACAAA
GGTCAAAGGT CATACAACAC ATGTGGATAA CAATGATATG GGTAACTTGA
TTGTGACGAA TAAATATACG CCAGAAACAA CATCAATTAG TGGTGAAAAA
GTATGGGACG ACAAAGACAA TCAAGATGGT AAGAGACCAG AAAAAGTCAG
TGTGAATTTA TTGGCTGATG GAGAGAAAGT AAAAACGTTA GACGTGACAT
CTGAAACAAA CTGGAAGTAC GAATTTAAAG ACTTACCGAA GTATGATGAA
GGAAAGAAAA TAGAATATAC AGTGACCGAA GATCACGTAA AAGACTACAC
AACAGACATC AACGGTACGA CAATAACGAA CAAGTATACA CCAGGAGAGA
CATCGGCAAC AGTAACAAAA AATTGGGATG ACAATAATAA CCAAGACGGA
AAACGACCAA CTGAAATCAA AGTTGAGTTA TATCAAGACG GAAAAGCAAC
AGGAAAAACG GCAACATTAA ATGAATCTAA TAACTGGACC CATACGTGGA
CAGGATTAGA TGAAAAAGCA AAAGGACAAC AAGTAAAATA CACAGTCGAG

10. Chemical synthesis method for producing a collagen binding protein or polypeptide according to claim 1, whereby an amino acid residue is built up based on said nucleotide sequence encoding for said protein or polypeptide starting from the C-terminal serine, which is stepwise reacted with the appropriate amino acid, whereby it is finally reacted with alanine at the N-terminal end, to form the collagen binding protein or polypeptide.

11. A collagen binding protein or polypeptide comprising at least one of the amino acid sequence

Ala
ArgAspIleSerSerThrAsnValThrAspLeuThrValSerProSerLysIleGluAsp
GlyGlyLysThrThrValLysMetThrPheAspAspLysAsnGlyLysIleGlnAsnGly
AspMetIleLysValAlaTrpProThrSerGlyThrValLysIleGluGlyTyrSerLys
ThrValProLeuThrValLysGlyGluGlnValGlyGlnAlaValIleThrProAspGly
AlaThrIleThrPheAsnAspLysValGluLysLeuSerAspValSerGlyPheAlaGlu
PheGluValGlnGlyArgAsnLeuThrGlnThrAsnThrLeuAspAspLysValAlaThr
IleThrSerGlyAsnLysSerThrAsnValIleGlyTrpIleLysValLysArgGluPro
ValValPheLeuIleAsnLysSerGlyLysIleCysTyrGlnGluAspThrThrHisVal
ArgTrpPheLeuAsnIleAsnAsnGluLysSerTyrValSerLysAspIleThrIleLys
AspGlnIleGlnGlyGlyGlnGlnLeuAspLeuSerThrLeuAsnIleAsnValThrGly
ThrHisSerAsnTyrTyrSerGlyGlnSerAlaIleThrAspPheGluLysAlaPhePro
GlySerLysIleThrValAspAsnThrLysAsnThrIleAspValThrIleProGlnGly
TyrGlySerTyrAsnSerPheSerIleAsnTyrLysThrLysIleThrAsnGluGlnGln
LysGluPheValAsnAsnSerGlnAlaTrpTyrGlnGluHisGlyLysGluGluValAsn
GlyLysSerPheAsnHisThrValHisAsnIleAsnAlaAsnAlaGlyIleGluGlyThr
ValLysGlyGluLeuLysValLeuLysGlnAspLysAspThrLysAlaProIleAlaAsn
ValLysPheLysLeuSerLysLysAspGlySerValValLysAspAsnGlnLysGluIle
GluIleIleThrAspAlaAsnGlyIleAlaAsnIleLysAlaLeuProSerGlyAspTyr
IleLeuLysGluIleGluAlaProArgProTyrThrPheAspLysAspLysGluTyrPro
PheThrMetLysAspThrAspAsnGlnGlyTyrPheThrThrIleGluAsnAlaLysAla
IleGluLysThrLysAspValSerAlaGlnLysValTrpGluGlyThrGlnLysValLys
ProThrIleTyrPheLysLeuTyrLysGlnAspAspAsnGlnAsnThrThrProValAsp
LysAlaGluIleLysLysLeuGluAspGlyThrThrLysValThrTrpSerAsnLeuPro
GluAsnAspLysAsnGlyLysAlaIleLysTyrLeuValLysGluValAsnAlaGlnGly

FIG. 1

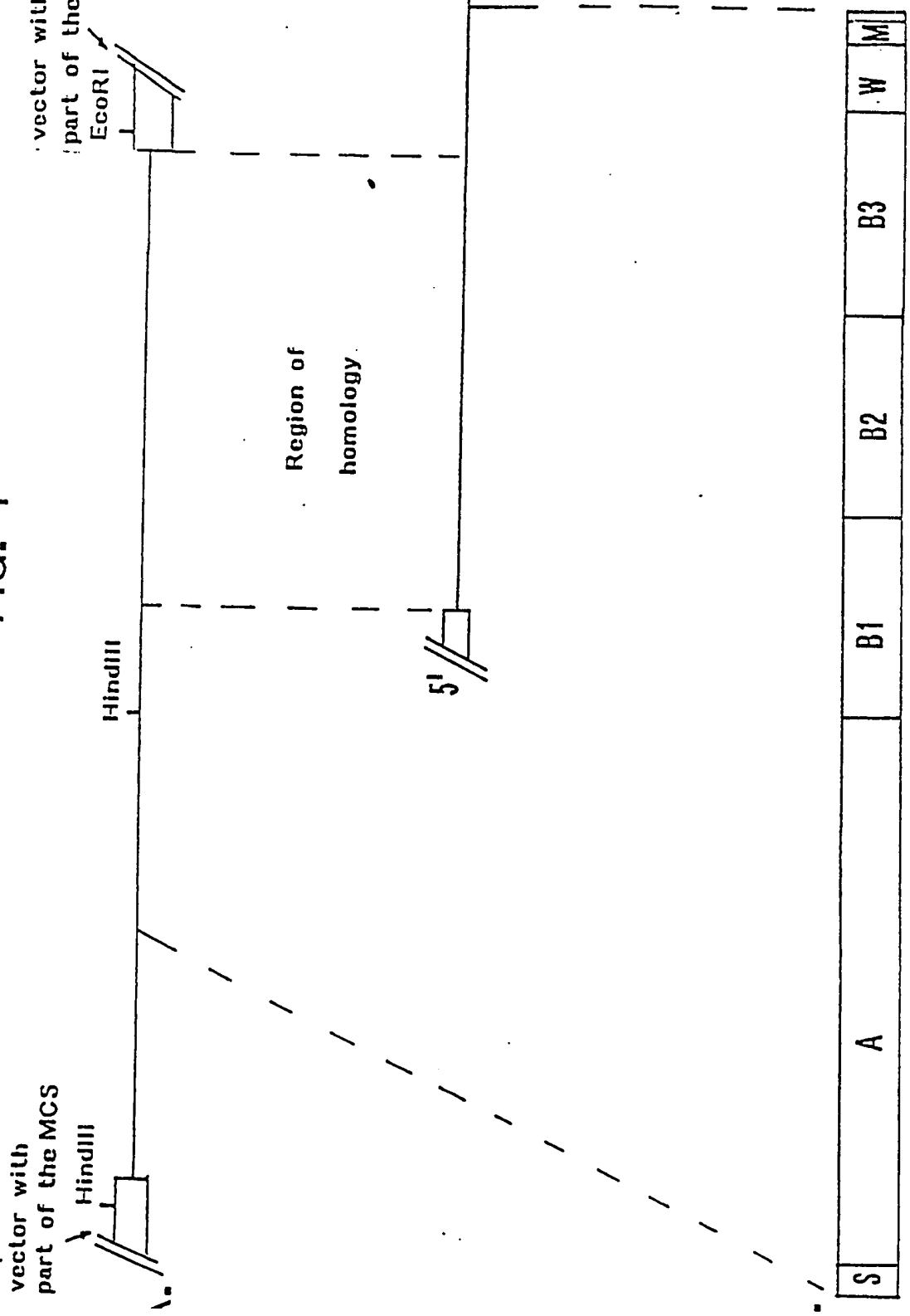
vector with part of the MCS
HindIII
vector with part of the MCS
EcoRI
p16
cCOL R6A
Region of homology
5'
S
A
B1
B2
B3
W
M

FIG. 2 (cont'd)

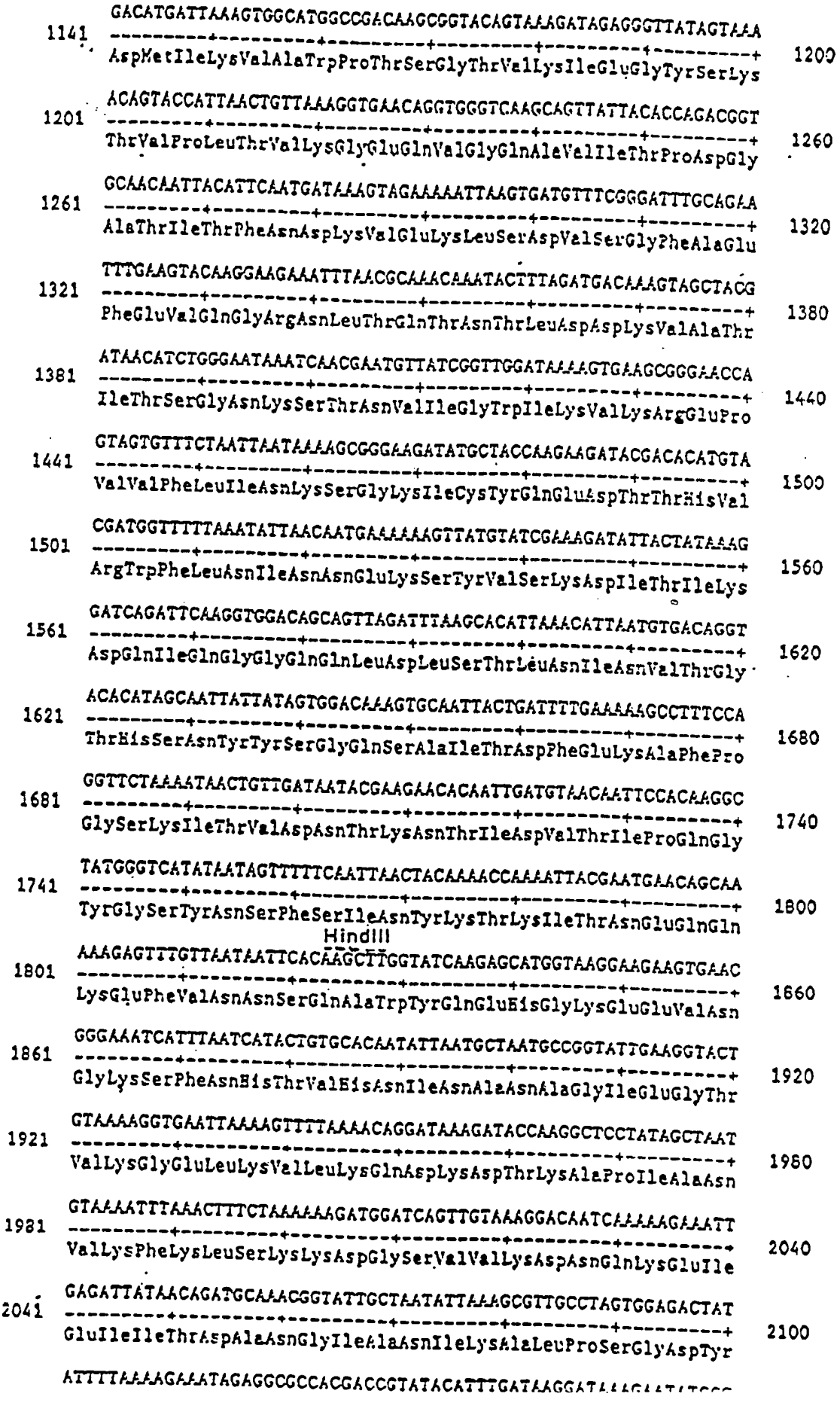
GACATGATTAAAGTGGCATGGCCGACAAGCGGTACAGTAAAGATAGAGGGTTATAGTAAA
1141
AspMetIleLysValAlaTrpProThrSerGlyThrValLysIleGluGlyTyrSerLys
1200
ACAGTACCATTAACTGTTAAAGGTGAACAGGTGGGTCAAGCAGTTATTACACCAGACGGT
1201
ThrValProLeuThrValLysGlyGluGlnValGlyGlnAlaValIleThrProAspGly
1260
GCAACAATTACATTCAATGATAAAGTAGAAAAATTAAGTGATGTTTCGGGATTTGCAGAA
1261
AlaThrIleThrPheAsnAspLysValGluLysLeuSerAspValSerGlyPheAlaGlu
1320
TTTGAAGTACAAGGAAGAAATTTAACGCAAACAAATACTTTAGATGACAAAGTAGCTACG
1321
PheGluValGlnGlyArgAsnLeuThrGlnThrAsnThrLeuAspAspLysValAlaThr
1380
ATAACATCTGGGAATAAATCAACGAATGTTATCGGTTGGATAAAAGTGAAGCGGGAACCA
1381
IleThrSerGlyAsnLysSerThrAsnValIleGlyTrpIleLysValLysArgGluPro
1440
GTAGTGTTTCTAATTAATAAAAGCGGGAAGATATGCTACCAAGAAGATACGACACATGTA
1441
ValValPheLeuIleAsnLysSerGlyLysIleCysTyrGlnGluAspThrThrHisVal
1500
CGATGGTTTTTAAATATTAACAATGAAAAAAGTTATGTATCGAAAGATATTACTATAAAG
1501
ArgTrpPheLeuAsnIleAsnAsnGluLysSerTyrValSerLysAspIleThrIleLys
1560
GATCAGATTCAAGGTGGACAGCAGTTAGATTTAAGCACATTAAACATTAATGTGACAGGT
1561
AspGlnIleGlnGlyGlyGlnGlnLeuAspLeuSerThrLeuAsnIleAsnValThrGly
1620
ACACATAGCAATTATTATAGTGGACAAAGTGCAATTACTGATTTTGAAAAAGCCTTTCCA
1621
ThrHisSerAsnTyrTyrSerGlyGlnSerAlaIleThrAspPheGluLysAlaPhePro
1680
GGTTCTAAAATAACTGTTGATAATACGAAGAACACAATTGATGTAACAATTCCACAAGGC
1681
GlySerLysIleThrValAspAsnThrLysAsnThrIleAspValThrIleProGlnGly
1740
TATGGGTCATATAATAGTTTTTCAATTAACTACAAAACCAAAATTACGAATGAACAGCAA
1741
TyrGlySerTyrAsnSerPheSerIleAsnTyrLysThrLysIleThrAsnGluGlnGln
1800
HindIII
AAAGAGTTTGTTAATAATTCACAAGCTTGGTATCAAGAGCATGGTAAGGAAGAAGTGAAC
1801
LysGluPheValAsnAsnSerGlnAlaTrpTyrGlnGluHisGlyLysGluGluValAsn
1860
GGGAAATCATTTAATCATACTGTGCACAATATTAATGCTAATGCCGGTATTGAAGGTACT
1861
GlyLysSerPheAsnHisThrValHisAsnIleAsnAlaAsnAlaGlyIleGluGlyThr
1920
GTAAAAGGTGAATTAAAAGTTTTAAAACAGGATAAAGATACCAAGGCTCCTATAGCTAAT
1921
ValLysGlyGluLeuLysValLeuLysGlnAspLysAspThrLysAlaProIleAlaAsn
1980
GTAAAATTTAAACTTTCTAAAAAAGATGGATCAGTTGTAAAGGACAATCAAAAAGAAATT
1981
ValLysPheLysLeuSerLysLysAspGlySerValValLysAspAsnGlnLysGluIle
2040
GAGATTATAACAGATGCAAACGGTATTGCTAATATTAAAGCGTTGCCTAGTGGAGACTAT
2041
GluIleIleThrAspAlaAsnGlyIleAlaAsnIleLysAlaLeuProSerGlyAspTyr
2100
ATTTTAAAAGAAATAGAGGCGCCACGACCGTATACATTTGATAAGGATAAAGAATATCCC

FIG. 2 - (cont'd)

```
                GTATGGGACGACAAAGACAATCAAGATGGTAAGAGACCAGAAAAAGTCAGTGTGAATTTA
3121            ---------+---------+---------+---------+---------+---------+ 3180
                ValTrpAspAspLysAspAsnGlnAspGlyLysArgProGluLysValSerValAsnLeu

                TTGGCTGATGGAGAGAAAGTAAAAACGTTAGACGTGACATCTGAAACAAACTGGAAGTAC
3181            ---------+---------+---------+---------+---------+---------+ 3240
                LeuAlaAspGlyGluLysValLysThrLeuAspValThrSerGluThrAsnTrpLysTyr

                GAATTTAAAGACTTACCGAAGTATGATGAAGGAAAGAAAATAGAATATACAGTCACCGAA
3241            ---------+---------+---------+---------+---------+---------+ 3300
                GluPheLysAspLeuProLysTyrAspGluGlyLysLysIleGluTyrThrValThrGlu

                GATCACGTAAAAGACTACACAACAGACATCAACGGTACGACAATAACGAACAAGTATACA
3301            ---------+---------+---------+---------+---------+---------+ 3360
                AspHisValLysAspTyrThrThrAspIleAsnGlyThrThrIleThrAsnLysTyrThr

                CCAGGAGAGACATCGGCAACAGTAACAAAAAATTGGGATGACAATAATAACCAAGACGGA
3361            ---------+---------+---------+---------+---------+---------+ 3420
                ProGlyGluThrSerAlaThrValThrLysAsnTrpAspAspAsnAsnAsnGlnAspGly

                AAACGACCAACTGAAATCAAAGTTGAGTTATATCAAGACGGAAAAGCAACAGGAAAAACG
3421            ---------+---------+---------+---------+---------+---------+ 3480
                LysArgProThrGluIleLysValGluLeuTyrGlnAspGlyLysAlaThrGlyLysThr

                GCAACATTAAATGAATCTAATAACTGGACCCATACGTGGACAGGATTAGATGAAAAAGCA
3481            ---------+---------+---------+---------+---------+---------+ 3540
                AlaThrLeuAsnGluSerAsnAsnTrpThrHisThrTrpThrGlyLeuAspGluLysAla

                AAAGGACAACAAGTAAAATACACAGTCGAGGAATTAACAAAGGTCAAAGGTTATACAACA
3541            ---------+---------+---------+---------+---------+---------+ 3600
                LysGlyGlnGlnValLysTyrThrValGluGluLeuThrLysValLysGlyTyrThrThr
                                                                  ↱ B3
                CATGTGGATAACAATGATATGGGCAACTTGATTGTGACGAATAAATATACGCCAGAAACA
3601            ---------+---------+---------+---------+---------+---------+ 3660
                HisValAspAsnAsnAspMetGlyAsnLeuIleValThrAsnLysTyrThrProGluThr

                ACATCAATTAGCGGTGAAAAAGTATGGGACGACAAAGACAATCAAGATGGTAAGAGACCA
3661            ---------+---------+---------+---------+---------+---------+ 3720
                ThrSerIleSerGlyGluLysValTrpAspAspLysAspAsnGlnAspGlyLysArgPro

                GAAAAAGTCAGTGTAAATTTATTGGCTAACGGAGAGAAAGTAAAAACGTTAGACGTGACA
3721            ---------+---------+---------+---------+---------+---------+ 3780
                GluLysValSerValAsnLeuLeuAlaAsnGlyGluLysValLysThrLeuAspValThr

                TCTGAAACAAACTGGAAGTACGAATTTAAAGACTTACCGAAGTATGATGAAGGAAAGAAA
3781            ---------+---------+---------+---------+---------+---------+ 3840
                SerGluThrAsnTrpLysTyrGluPheLysAspLeuProLysTyrAspGluGlyLysLys

                ATAGAATATACAGTGACCGAAGATCACGTAAAAGACTACACAACAGACATCAACGGTACG
3841            ---------+---------+---------+---------+---------+---------+ 3900
                IleGluTyrThrValThrGluAspHisValLysAspTyrThrThrAspIleAsnGlyThr
                  3' end of insert in p16 ←┐
                ACAATAACGAACAAGTATACACCAGGAGAGACATCGGCAACAGTAACAAAAAATTGGGAT
3901            ---------+---------+---------+---------+---------+---------+ 3960
                ThrIleThrAsnLysTyrThrProGlyGluThrSerAlaThrValThrLysAsnTrpAsp

                GACAATAATAACCAAGACGGAAAACGACCAACTGAAATCAAAGTTGAGTTATATCAAGAT
3961            ---------+---------+---------+---------+---------+---------+ 4020
```

FIG. 3

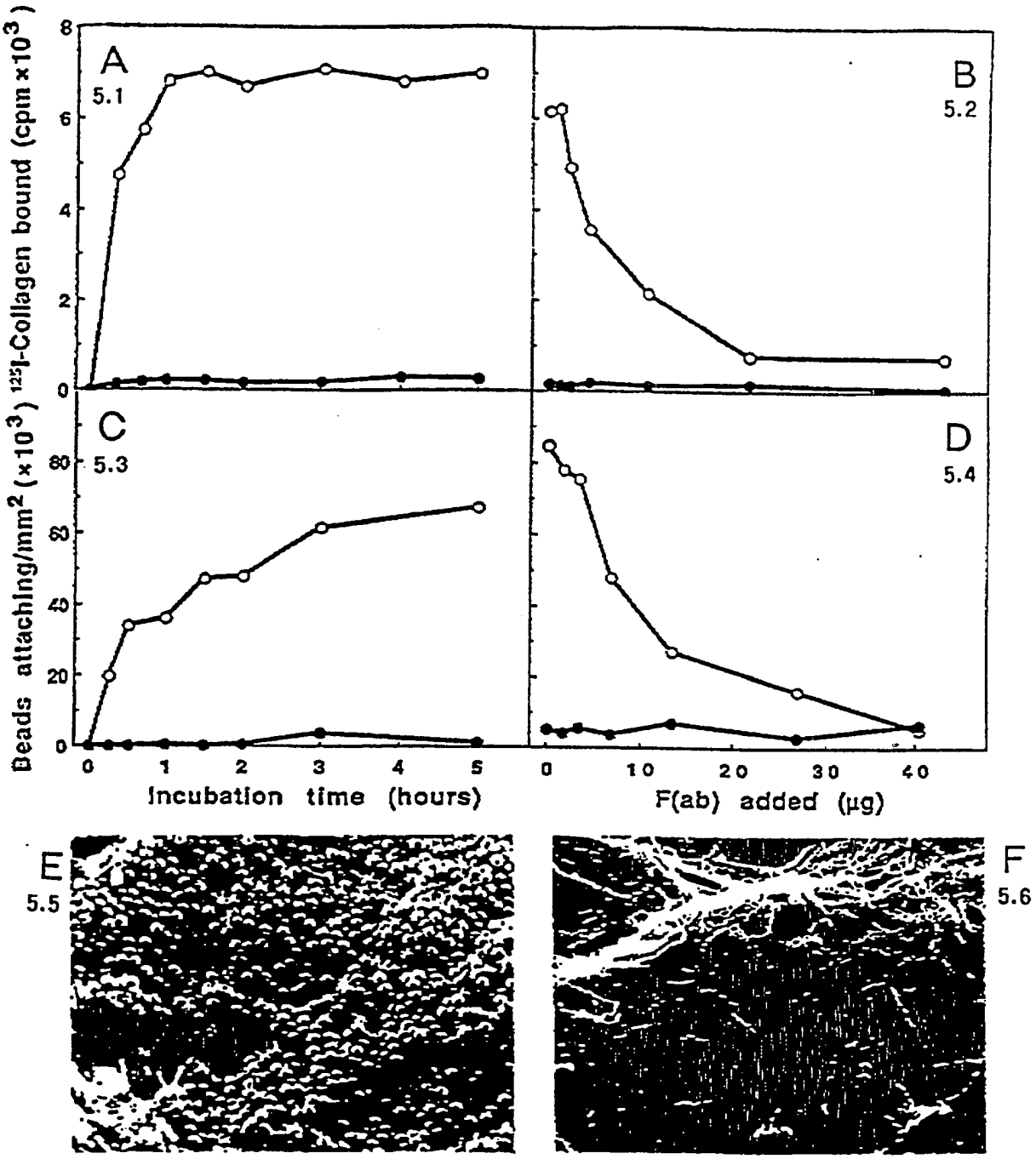
A
5.1
B
5.2
C
5.3
D
5.4
Beads attaching/mm² (×10³) ¹²⁵I-Collagen bound (cpm ×10³)
Incubation time (hours)
F(ab) added (µg)
E
5.5
F
5.6

FIG. 5

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 91/00707

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/04

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 07 K; C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP, A2, 0163623 (ALFA-LAVAL AGRI INTERNATIONAL AB) 4 December 1985, see page 8 lines 7-15 --	1-11
X	Dialog Information Services, file 55: BIOSIS 85-92, Dialog accession no. 7397648, Switalski L M et al: "Isolation and characterization of a putative collagen receptor from staphylococcus--aureus strain cowan 1", & J Biol Chem 264 (35) 1989 21080-21086, -- --------	1-11

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
3rd February 1992	1992 -02- 06
International Searching Authority	Signature of Authorized Officer